



Highly efficient one-step PCR-based mutagenesis technique for large plasmids using high-fidelity DNA polymerase

H. Liu, R. Ye and Y.Y. Wang

Department of Medical Microbiology and Parasitology,
School of Basic Medical Sciences, Fudan University, Shanghai, China

Correspondence author: Y.Y. Wang
E-mail: yuyanss@fudan.edu.cn

Genet. Mol. Res. 14 (2): 3466-3473 (2015)
Received March 21, 2014
Accepted October 11, 2014
Published April 15, 2015
DOI <http://dx.doi.org/10.4238/2015.April.15.10>

ABSTRACT. Gene mutation plays an important role in molecular biological studies. A highly efficient one-step polymerase chain reaction-based mutagenesis technique for site-directed mutagenesis was developed in this study. One complementary pair of primers was designed that contained the desired mutations in the middle of the primers. The amplification products of mutation were amplified using a high-fidelity DNA polymerase and the original plasmid templates were digested by *DpnI*. This method was successfully used to introduce mutations in two different-sized plasmids (12 and 6 kb) with high efficiency. The results indicate that this technique can be widely used to introduce any plasmid mutations quickly and efficiently.

Key words: *DpnI* digestion; High-fidelity DNA polymerase; Site-directed mutagenesis

INTRODUCTION

Site-directed mutagenesis is the foundation of modern molecular biology and genetic engineering and is widely used to study protein structure-function relationships. Directed mutagenesis includes adding and deleting bases as well as point mutations by changing a particular nucleotide sequence to precisely alter the protein structure. Currently, site-directed mutagenesis involves non-polymerase chain reaction (PCR)- and PCR-based techniques (Gillam et al., 1975; Zoller and Smith, 1987; Qi and Scholthof, 2008; Wu et al., 2013). PCR-mediated mutagenesis techniques are simple, fast, and efficient, and the *Taq* DNA polymerase that is typically used in PCR can result in mismatch, affecting the accuracy of mutation (Shinde et al., 2003).

Currently, the most widely used strategy for introducing mutations is the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Using this method, the vector containing the mutated bases is amplified by one pair of completely complementary primers, and then the methylated DNA template is extracted from *Escherichia coli* and digested by the restriction enzyme *DpnI*. The PCR products that do not contain methylation sites cannot be digested by *DpnI*, and thus the products can be directly transformed into *E. coli* to obtain the desired vector containing the point mutation. High-fidelity *Taq* polymerase is typically used in this procedure. Though kits are convenient and involve simple operation steps, they are also expensive. In this study, some of the reagents and reaction conditions were modified and optimized to establish a simple, rapid, and economical method of site-directed mutagenesis.

In our protocol, a high-fidelity DNA polymerase to PCR-amplify the mutated product was used, and other reagents included routine laboratory reagents. Q5™ High-fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) is a high-fidelity, thermostable DNA polymerase with 3'→5' exonuclease activity, fused to a processivity-enhancing Sso7d domain to support robust DNA amplification. Because of its low error rate, Q5 High-fidelity DNA polymerase is ideal for cloning and can be used for long or difficult amplicons. Using Q5™ High-fidelity DNA polymerase, we successfully amplified gene sequences up to 12 kb using site-directed mutagenesis. In addition, we compared the method with the *Taq* DNA polymerase from another company to determine error rates, costs, and DNA product quality. Our data suggest that the Q5™ High-fidelity DNA polymerase method we developed is an excellent alternative for site-directed mutagenesis for long DNA fragments up to 12 kb.

MATERIAL AND METHODS

Preparation of template plasmid

The pMH54-cmS, a 12-kb plasmid inserted by S gene fragments of canine coronavirus, pIR-tN-EGFP (6 kb) inserted by N gene fragments of transmissible gastroenteritis virus, were used as the mutagenesis templates. The 2 plasmids were propagated in DH5α *E. coli* cells (Tiangen Biotech, Beijing, China), and prepared using Qiagen miniprep kits (Qiagen, Hilden, Germany).

Primer design

The 3 pairs of mutagenic oligonucleotide primers used in this study are listed in Table 1, which were designed to amplify the mutant fragments. These primer pairs contained com-

pletely complementary sequences, with mutated bases at the center position. Three different positions from the two different plasmids were selected for mutagenesis by primer design. The primer pairs used in this experiment were completely complementary. Primers tN-501F, tN-501R, tN-959F, and tN-959R were used to amplify the corresponding fragment for mutant pIR-tN⁵⁰¹-EGFP and pIR-tN⁹⁵⁹-EGFP, while MH-1700F and MH-1700R were designed for pMH54-cmS¹⁷⁰⁰ (Figure 1). All primers were synthesized and purified by Sangon Biotech (Shanghai, China). The NEB Tm Calculator was used to determine the optimal annealing temperatures for the primers.

Table 1. Primer sequences used in this study.

Primer name	Sequence (5'→3')
tN-501F	ACGCTCTCAATCTAG <u>A</u> TCTCGGTCTAGAAAT
tN-501R	ATTTCTAGACCGAG <u>A</u> ICTAGATTGAGAGCGT
tN-959F	ATGATCCTAAGACTG <u>A</u> ACAATTCCTTCAGCA
tN-959R	TGCTGAAGGAATTG <u>T</u> TTCAGTCTTAGGATCAT
MH-1700F	TAGCTGTGTTGATT <u>A</u> TTGTGTTTTTGATG
MH-1700R	CATCAAAAACACAA <u>A</u> ATCAACACAGCTA

Underlined letters are the mutated bases.

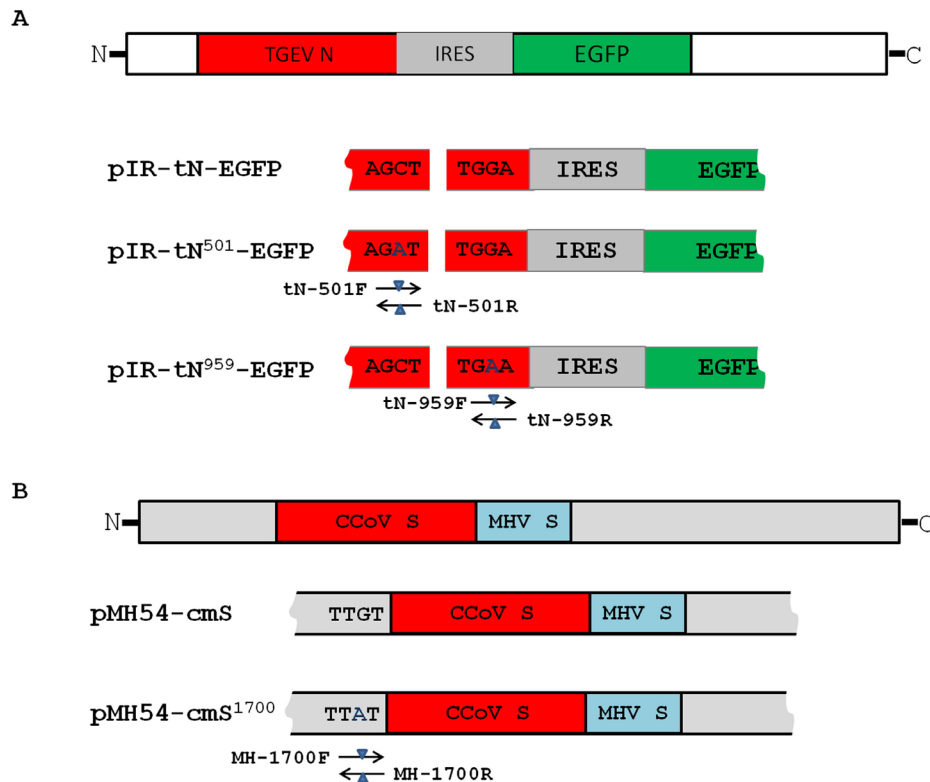


Figure 1. Schematic diagram of the primer design for site-directed mutagenesis. **A.** tN-501F, tN-501R, tN-959F, and tN-959R were for pIR-tN-EGFP. **B.** MH-1700F and MH-1700R were designed for pMH54-cmS¹⁷⁰⁰.

PCR amplification of target-mutated fragment

The PCR mixture (50 μ L) contained 1 ng template, 0.5 μ M primer pair, 200 μ M dNTPs, 10 μ L 5X Q5 reaction buffer, and 1 U Q5TM High-fidelity DNA polymerase (New England Biolabs). The reaction started with template pre-denaturation at 98°C for 30 s, followed by 25 cycles at 98°C for 10 s, 55°C for 1 s, and 72°C for 2 or 4 min according to the length of the template constructs (about 20-30 s/kb), and then an extension step at 72°C for 5 min. PCR amplifications were confirmed by 1% agarose gel electrophoresis, and specific bands were observed under ultraviolet light. The reaction solution was stored at 4°C. Using these same steps, we amplified a mutated fragment using *Taq* DNA polymerase (New England Biolabs).

Digestion of template and transformation of digestion products

PCR products were treated with the restriction enzyme *DpnI* (New England Biolabs) at 37°C for 2 h to digest the plasmid template. Next, 10 μ L digestion products were transformed into DH5 α *E. coli* cells by heat shock. The transformed mix was spread and inoculated onto Luria-Bertani plates containing kanamycin or ampicillin and then incubated at 37°C overnight. The number of colonies was counted to determine the number of colony forming units (CFU). Six colonies from each plate were grown in Luria-Bertani overnight and the plasmid DNA was extracted using Qiagen miniprep kits.

Verification of plasmids

The length of the plasmid was identified by restriction enzyme digestion. The introduction of site-directed mutagenesis was evaluated by plasmid DNA sequencing (Invitrogen, Carlsbad, CA, USA).

RESULTS

Detection of gene mutation by PCR

A large plasmid pMH54-cmS (12 kb) and common plasmid pIR-tN-EGFP (6 kb) were used as template plasmids in this study. The full-length pMH54-cmS¹⁷⁰⁰ fragment (12.3 kb) obtained by PCR is shown in Figure 2A (lane 3). Figure 2A (lanes 1 and 2) shows the PCR screening for point mutations (S167R and G320E) in pIR-tN-EGFP.

Counting of transformed colonies

The plate was coated with 1 mL bacteria, and colonies were counted after incubation overnight. The number of pMH54-cmS¹⁷⁰⁰ colonies was 2.7 CFU/mL, while the number of pIR-tN⁵⁰¹-EGFP was 37.8 CFU/mL and 52.5 CFU/mL for pIR-tN⁹⁵⁹-EGFP (Figure 2B).

Verification of plasmid mutants

Six clones per plasmid mutant were extracted and showed the expected sizes (Figure 3A). Plasmids pIR-tN⁵⁰¹-EGFP and pIR-tN⁹⁵⁹-EGFP were subjected to restriction enzyme di-

gestion (*AvrII*), generating 3 fragments (3.1, 2.4, 1.0 kb) (Figure 3B). Plasmid pMH54-cmS¹⁷⁰⁰ was digested by *EcoRI* and *PstI*, generating 4 fragments (1.4, 2.7, 3.4, 4.7 kb) (Figure 3B).

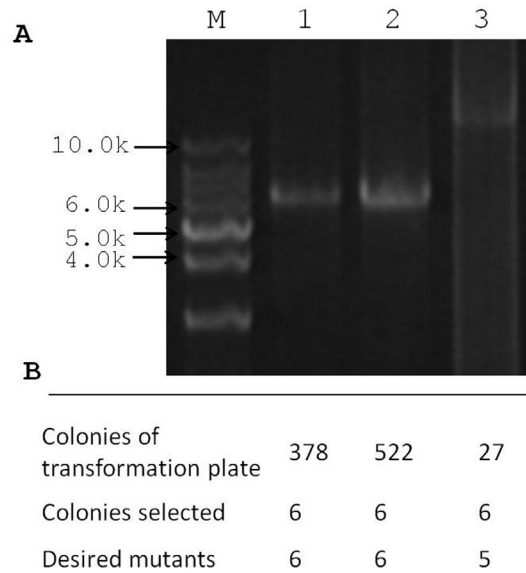


Figure 2. PCR amplification for single-site mutagenesis. **A.** Agarose gel electrophoresis of the PCRs indicating the amplification efficiency. *Lane 1* was for pIR-tN501-EGFP, *lane 2* was for pIR-tN959-EGFP, and *lane 3* was for pMH54-cmS1700. *Lane M* = DNA marker. **B.** Transformation and mutation efficiency of each reaction for pIR-tN501-EGFP, pIR-tN959-EGFP, and pMH54-cmS1700.

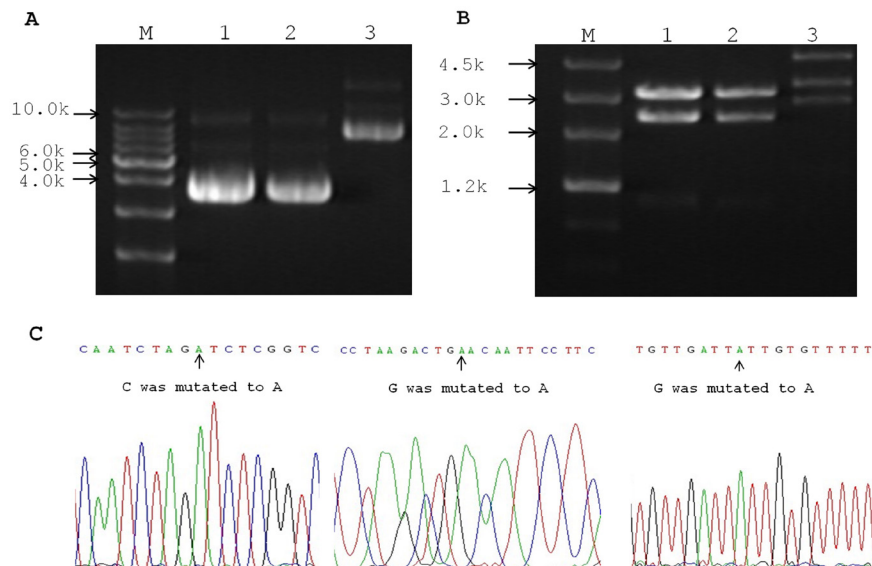


Figure 3. Verification of plasmid mutants. **A.** Agarose gel electrophoresis of three plasmid mutants. **B.** Restriction digestion of plasmids. *Lane 1* = pIR-tN⁵⁰¹-EGFP was digested by *AvrII*; *lane 2* = pIR-tN⁹⁵⁹-EGFP was digested by *AvrII*; *lane 3* = pMH54-cmS¹⁷⁰⁰ was digested by *EcoRI* and *PstI*. **C.** Sequencing results of site-directed saturation mutagenesis.

A total of 18 colonies were analyzed by sequencing (Figure 3C), only 1 of which was a sequencing error compared to the theoretical sequence. The success rate of site-directed mutagenesis using this protocol was 94.4%, while the success rate of site-directed mutagenesis for the normal size plasmid (6.4 kb) was 100% and that of the large plasmid (12.3 kb) was 83.3%.

DISCUSSION

Amplification efficiency of Q5™ High-fidelity DNA polymerase

In this experiment, we found that Q5™ DNA polymerase was efficient in the PCR amplification. The *Taq* DNA polymerase from New England Biolabs used for PCR only amplified a small fragment of 2 kb, but was ineffective for long plasmids such as pMH54-cmS and pIR-tN-EGFP (data not shown). We found that the mutation products could be detected by agarose gel electrophoresis, while the QuikChange™ Site-Directed Mutagenesis System recommended continuing to the next step regardless of whether a visible fragment was detected by agarose gel electrophoresis. A visible fragment can increase the number of positive clones and increase the successes and failures of the procedure. In addition, other fidelity DNA polymerases produced by different companies have been reported to substitute and compared with QuikChange™, but whether 12 kb can be amplified is unknown (Picard et al., 1994; Liang et al., 1995; Adereth et al., 2005; Wu et al., 2013).

Accuracy and mutation rate of Q5™ High-fidelity DNA polymerase

Q5™ High-fidelity DNA polymerase possesses 3'→5' exonuclease activity, which can remove mismatched nucleotides during PCR. It is also fused to a processivity-enhancing Sso7d domain to support robust DNA amplification. Q5™ DNA polymerase is supplied with an optimized buffer system to allow for robust amplification regardless of GC content. The 5X Q5 Reaction Buffer contains 2 mM MgCl₂ at final (1X) reaction concentration and is recommended for most routine applications. For GC-rich targets (≥65% GC), amplification can be improved by adding 5X Q5 High GC Enhancer. Q5™ High-fidelity DNA polymerase is unlike typical, lower fidelity PCR enzymes. Q5™ High-fidelity DNA polymerase has high fidelity with an error rate that is 100-fold lower than that of *Taq* DNA polymerase and 12-fold lower than that of *Pyrococcus furiosus* (PFU) DNA polymerase (NEB Information).

Our results demonstrate that the success rate of site-directed mutagenesis for the plasmid of typical size (6.4 kb) was 100, while this value was 83.3% for a large plasmid (12.3 kb), with high fidelity and amplification efficiency by Q5™ DNA polymerase to introduce mutations. We ensured the high efficiency by increasing the template concentration, reducing the number of PCR cycles, using a unique mutant primer design.

DpnI is used to digest the plasmid template, removing the non-mutated background. *DpnI* treatment can be used to obtain the correct mutant plasmid. Nearly all *E. coli* contain a gene encoding a 6-purine methylase, which methylates the 6-purine GATC sequence. *DpnI* can recognize methylated GATC and cut both methylated and hemi-methylated DNA (Shenoy and Visweswariah, 2003; Li et al., 2008). DNA synthesized *in vitro* is unmethylated, and thus can be selectively digested, while the newly synthesized PCR products are unaffected. To ensure complete template digestion, the digestion time could be extended to 2-3 h or more or the amount of *DpnI* can be increased (10-20 U). The same procedure without PCR primer

can be conducted to evaluate whether *DpnI* treatment was effective. If the plasmid template is completely digested by *DpnI*, the control plate that did not include primer in the PCR should show colonies.

Simplicity and rapidity of the site-directed mutagenesis technique

Our new protocol was successfully used to amplify mutation fragments of different lengths in one step and is simple and easy to perform (Figure 4). The completely complementary mutagenic primer only annealed to and extended the original template in the cycle, such that Q5™ DNA polymerase showed a linear amplification and not exponential amplification observed in typical PCR. This method ensures the greatest accuracy of template replication during temperature cycling. The residual template was digested by *DpnI*, while the sub-chain with mutation forms double-stranded DNA containing a nick. The products were transformed into *E. coli* cells where the nick was ligated by host repair enzymes, and DH5α *E. coli* cells were the most effective for the large plasmid. The entire process can be completed in only 1 day.

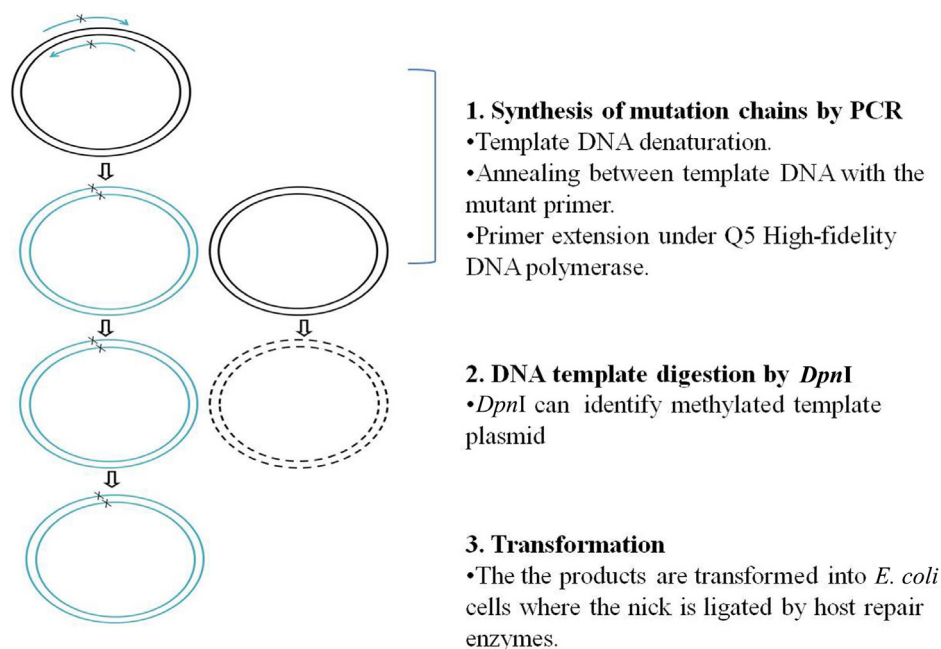


Figure 4. Schematic presentations using the one-step method.

We developed an economic method requiring only a single PCR amplification step. The Q5™ DNA polymerase and *DpnI* must be purchased but are inexpensive, and other reagents include common laboratory reagents. The method is simple and convenient; the primer design requirements are simple, the process is easy to operate, and the entire process can be completed in only 1 day. The protocol is widely applicable for laboratory gene mutagenesis transformation, and is suitable for plasmids with a wide range of sizes, including 6-12 kb.

ACKNOWLEDGMENTS

Research supported by open funds of the State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (#SKLVEB2011KFKT003).

REFERENCES

- Adereth Y, Champion KJ, Hsu T and Dammai V (2005). Site-directed mutagenesis using Pfu DNA polymerase and T4 DNA ligase. *Biotechniques* 38: 864-868.
- Gillam S, Waterman K and Smith M (1975). Enzymatic synthesis of oligonucleotides of defined sequence. Addition of short blocks of nucleotide residues to oligonucleotide primers. *Nucleic Acids Res.* 2: 613-624.
- Li J, Li C, Xiao W, Yuan D, et al. (2008). Site-directed mutagenesis by combination of homologous recombination and *DpnI* digestion of the plasmid template in *Escherichia coli*. *Anal. Biochem.* 373: 389-391.
- Liang Q, Chen L and Fulco AJ (1995). An efficient and optimized PCR method with high fidelity for site-directed mutagenesis. *PCR Methods Appl.* 4: 269-274.
- Picard V, Ersdal-Badju E, Lu A and Bock SC (1994). A rapid and efficient one-tube PCR-based mutagenesis technique using Pfu DNA polymerase. *Nucleic Acids Res.* 22: 2587-2591.
- Qi D and Scholthof KB (2008). A one-step PCR-based method for rapid and efficient site-directed fragment deletion, insertion, and substitution mutagenesis. *J. Virol. Methods* 149: 85-90.
- Shenoy AR and Visweswariah SS (2003). Site-directed mutagenesis using a single mutagenic oligonucleotide and *DpnI* digestion of template DNA. *Anal. Biochem.* 319: 335-336.
- Shinde D, Lai Y, Sun F and Arnheim N (2003). Taq DNA polymerase slippage mutation rates measured by PCR and quasi-likelihood analysis: (CA/GT)_n and (A/T)_n microsatellites. *Nucleic Acids Res.* 31: 974-980.
- Wu D, Guo X, Lu J, Sun X, et al. (2013). A rapid and efficient one-step site-directed deletion, insertion, and substitution mutagenesis protocol. *Anal. Biochem.* 434: 254-258.
- Zoller MJ and Smith M (1987). Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. *Methods Enzymol.* 154: 329-350.